

RECOMBINANT IMMUNOGENS FOR THE GENERATION OF
ANTIVENOMS TO THE VENOM OF SCORPIONS OF THE GENUS
CENTRUROIDES

[0001] This application claims the benefit of priority of U.S. Provisional Application No. 60/430,067 filed December 2, 2002 which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to the isolated genes that code for scorpion toxins (particularly, toxins that affect the sodium and Erg type potassium channels), to fusions of said genes with fragments of genes from other proteins, to the genetic constructions they comprise, to the isolated recombinant fusion proteins and recombinant peptides that comprise the primary sequence of the toxins in question, to their use as immunogens or antigens for the generation of antibodies that can recognize and neutralize components of the scorpion venom from which the toxin genes were obtained, to their use as vaccines to prevent envenomation from stings of scorpions of the genus *Centruroides*, and to their use as part of an immunogenic matrix for the purification of specific immunoglobulins.

[0003] Scorpion venom is a complex mixture of peptides, which can be classified in two groups based on the number of amino acids: long chain toxins with 60-76 amino acids (Possani, L.D., *et al.*, *Eur. J. Biochem.* 264:287-300 (1999)), which block the Na⁺ channels of excitable cells and short chain toxins with 29-41 amino acids that affect the K⁺ channels (Possani, L.D., *et al.*, *Biochemie* 82:861-868 (2000)), including the Erg type channels (Ether a go go).

[0004] The toxins that modify the Na⁺ channels are, in turn, classified as α and β toxins (Couraud, F., *et al.*, *Toxicon* 20:9-16 (1982)). The α -toxins mainly modify the inactivation mechanism of the Na⁺ channels, while the β -toxins

preferentially modify their activation mechanism (Strichartz, G., *et al.*, *Annu. Rev. Neurosci* 10:237-267 (1987)).

[0005] Scorpion venom is composed of several peptides that can be toxic for a large variety of animals, some are toxic for mammals, others for insects, others for crustaceans, etc. (Possani, L.D., *et al.*, *Eur. J. Biochem.* 264:287-300 (1999)).

[0006] There are approximately 221 species and subspecies of scorpions in Mexico, but only eight are medically important and are those that belong to the genus *Centruroides*, of which the species *Centruroides noxius* Hoffmann, *C. limpidus limpidus* Krash, *C. elegans* and *C. sculpturatus* are some of the most dangerous (Calderón-Aranda, *et al.*, *Toxicon* 31:327-337 (1993)). From 1981 to 1990, approximately 250,000 cases of stings in humans were reported, with some 300 deaths (Calderón -Aranda, E.S., *et al.*, *Vaccine* 13:1198-1206 (1995)), while from 1996-1998, of the cases that were reported among the population covered by the Mexican Social Security Institute, there were 429,561 cases of intoxication caused by scorpion stings (Esteba Maraboto, J. A., and Turrubiate Guillén, N. (1999). Panorama epidemiológico de las intoxicaciones causadas por animales ponzoñosos en la población derechohabiente del IMSS 1990-1998.5ª Reunión de expertos en Envenenamiento por Animales Ponzoñosos. Instituto de Biotecnología/UNAM Cuernavaca, Morelos, Mexico, 17 February, 1999). Hence scorpionism is considered to be a public health problem in Mexico.

[0007] In the United States, although the incidence of scorpion stings is much lower than in Mexico, there is a health problem caused by the scorpion *C. sculpturatus* of Arizona. This low incidence of cases of envenomation from this scorpion means a very small potential market, which has discouraged the large pharmaceutical companies from developing and producing efficacious antivenoms, and therefore cases are usually attended with intensive therapy, that is, relieving the symptoms caused by means of intensive care. In general, symptoms following a scorpion sting include: pain, cough, hypersensitivity, hyperexcitability, excessive salivation and vomiting. When two or more of

these symptoms are present, antiscorpion serum is administered to counteract the effects, since cases of severe envenomation can cause heart and/or lung failure (Nonner, W., *Adv. Cytopharmacol.* 3:345-351 (1979)), which can cause the death of the individual who has been stung.

[0008] Perhaps the most successful strategy in treating scorpion envenomation is the use of hyperimmune sera or monoclonal antibodies that can neutralize or delay the toxic effect of the scorpion toxins (serotherapy). In this sense, serotherapy studies have been conducted with polyclonal antibodies (Dehesa-Dávila, M., and Possani, L.D., *Toxicon* 32: 1015-1018 (1994)) and monoclonal antibodies (Dehesa-Dávila, M., et al., "Cinical toxicology of scorpion stings." In: *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, pp. 201-238 (Meier, J. and White, J. Eds.). New York, CRC Press (1995); Zamudio, F., et al., *Eur. J. Biochem.* 204:281-292 (1992)); similarly, there are products on the market obtained from hyperimmune anti-scorpion horse sera, that after being fractionated, are enzymatically digested to obtain the F(ab)₂ fragment (for example, the one prepared by Instituto Bioclón S.A., in Mexico). However, these sera are prepared by immunizing horses with the macerated telson of a large number of scorpions. Once this maceration has been prepared, it is centrifuged and the horse is inoculated with the soluble part. This procedure is carried out by macerating a mixture of telsons from several scorpion species, such as *C. noxius*, *C. limpidus limpidus* and *C. suffusus suffusus* (in Mexico) (Dehesa-Dávila, M., and Possani, L.D., *Toxicon* 32: 1015-1018 (1994)).

[0009] After several immunizations, the horse serum is obtained and whole immunoglobulins of the horse are purified. The antibodies are subsequently digested with pepsin to obtain the immunoglobulin fragments, F(ab)₂, responsible for neutralizing the venom.

[0010] The preparation described above has the disadvantage of containing a mixture of very varied antibodies, since, as mentioned earlier, scorpion venom is composed of dozens of peptides, only a few of which are active against mammals. Hence, the antibodies directed against these toxins are found in a

very small proportion with respect to the total population of antibodies, meaning that it is necessary to apply a high dose of the preparation in order to be able to neutralize the effect of the toxins. Since this is an exogenous protein, the larger the amount the greater the risk of provoking an anaphylactic shock or serum sickness in patients, and the greater the possibility of provoking secondary effects in persons to whom this neutralizing preparation is applied on more than one occasion.

[0011] Due to the above, there is a need to remove the immunoglobulins not associated with the neutralizing effect of these sera, since their administration can produce an undesirable immune response (crossed autoimmunity, for example), induce nephrotoxicity, serum sickness and, in serious cases, anaphylactic shock.

[0012] In the case of the use of monoclonal antibodies, there are other disadvantages such as the presence of pollutants in the culture mediums that contain antibodies expressed by a hybridoma of interest, such as cells or nucleic acids. In the same way, antibody aggregates can also act as immunogens and cause an undesirable immune response in the organism receiving the therapy.

[0013] Together with the failed attempts to generate a reliable vaccine, and until more adequate, safe peptides for vaccination are determined, the most viable alternative with greatest purity, at least in the short term, for protection from scorpion stings is the use of anti-scorpion fabotherapics.

[0014] Furthermore, independent of whether or not adequate vaccines are produced to prevent intoxication from scorpion venom, there will be the constant need to have an effective reagent (anti-scorpion antiserum or purified anti-scorpion antibodies) for administration to non-vaccinated, intoxicated individuals available for immediate use in the field, since the time in which the venom exerts its toxic effect and can even provoke death in the affected organism is very short (0.33 hrs. in mice) (Zamudio, F., *et al.*, *Eur. J. Biochem.* 204:281-292 (1992)).

[0015] For this reason, there is interest in constantly improving the production of the anti-scorpion antivenoms to be administered in order to neutralize the toxic activity of the scorpion venom in affected individuals.

[0016] Legros et al. (2002) published a report in which clones of mammal specific toxins I, II and III of *Androctonus australis* were used to produce recombinant peptides fused to the maltose binding protein (MBP). The fusion proteins were subsequently employed to generate antibodies in rabbits and proved to have a neutralizing effect on the toxic fraction (for mammals) that was separated from the scorpion venom, producing a sustained response. Hence, it is suggested that these recombinant peptides can even be used as a vaccine against the sting of this scorpion.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Figure 1. Shows the construction for the expression of recombinant peptide Cn5. A) shows the scheme of the expression vector pMal-C where the insert corresponding to clone CngtII is shown. B shows the information of the polylinker of the expression vector pMal-C, including the positions recognized by the restriction enzymes and factor Xa, and the position where CngtII was inserted.

[0018] Figure 2. Shows a 10% SDS PAGE gel dyed with Coomassie blue. Lanes 1, 2 and 3 contain the cell extracts of non-transformed, transformed but not induced and transformed and induced *E. coli*, respectively. The fusion protein FP obtained after purification by affinity chromatography using the extract of transformed, induced *E. coli* cells is in lane 4. Lane 5 shows fusion protein (FP) after digestion by factor Xa giving rise to the maltose binding protein and the recombinant peptide Cn5. Native toxin Cn5 is in lane 6. Lane 7 depicts the molecular weight markers that are indicated to the right of the figure.

[0019] Figure 3. Shows a western blot developed with anti native toxin Cn5 rabbit serum. The contents of the lanes are the same as in Figure 2.

[0020] Figure 4. Titration by ELISA of anti-native toxin Cn5 and anti- fusion protein (FP) sera, obtained from immunized rabbits. The open boxes correspond to titration of the anti Cn5 serum and the open circles correspond to titration of anti-FP serum.

DETAILED DESCRIPTION OF THE INVENTION

[0021] One way of improving the antivenoms being currently produced is to enrich the mixtures of scorpion venoms that are used as immunogens or antigens with selected toxins whose effect is known as being especially toxic for mammals. To this end, it would be necessary to purify large amounts of toxins from large amounts of venom. One interesting alternative is to substitute these native toxins with synthetic peptides or peptides produced using recombinant DNA techniques. Although feasible, chemical synthesis of the peptide is not economically recommendable on a large scale, while to be able to produce said recombinant peptides it is necessary to have the nucleotide sequences coding for these toxins.

[0022] It may prove even better to prepare a chemical composition consisting of a mixture of those toxins specific for mammals or alternatively a mixture of synthetic or recombinant peptides that have the same primary sequence as said toxins for use as an immunogen instead of the whole venoms for the generation of antibodies in mammals. It is postulated that said mixture of antibodies would have a clear advantage over those currently produced, since it would have been generated only against mammal specific toxins and would have greater venom neutralizing activity per milligram of exogenous protein administered to the organism affected by the venom.

[0023] Another way of improving the antivenoms currently produced is to separate those antibodies or their fragments that really participate in the neutralization of mammal specific toxins. To this end, it would be useful to have an antigenic matrix to which those antibodies against said toxins are specifically bound (immunoaffinity). This can be achieved by binding said isolated toxins to an inert material by way of support. Again, it is necessary to

have sufficient amounts of said toxins or, alternatively, recombinant or synthetic peptides with the same primary sequence as the toxins in question.

[0024] In order to be able to have sufficient amounts of peptides with the same primary sequence as the toxins that are potentially specific for mammals, which can be used to improve the antivenoms currently produced as mentioned in the paragraphs above, the inventors of the present invention decided to isolate and sequence several cDNA clones of toxins from different scorpions of potential interest to health in several regions, such as *Centruroides exilicauda*, *C. limpidus limpidus* Karsh, *C. noxius* Hoffmann, *C. elegans* and *C. gracilis* from Mexico and *C. sculpturatus* Ewing from the United States. The isolation and cloning of these genes is described in detail in examples 1 to 7. As shown in Table 1, it was possible to isolate and clone a total of 71 clones of toxins from 6 species of scorpions of the genus *Centruroides*, 49 of which are specific for sodium channels and 22 for Erg type potassium channels.

TABLE 1. 71 clones of genes of toxins isolated from scorpions of the genus *Centruroides*

Species	A	A1	B	C	D	E
<i>C. exilicauda</i>	Cex1	1	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 4
<i>C. exilicauda</i>	Cex2	1	SEQ ID NO: 5	SEQ ID NO: 6	SEQ ID NO: 7	SEQ ID NO: 8
<i>C. exilicauda</i>	Cex12	1	SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11	SEQ ID NO: 12
<i>C. exilicauda</i>	Cex13	1	SEQ ID NO: 13	SEQ ID NO: 14	SEQ ID NO: 15	SEQ ID NO: 16
<i>C. exilicauda</i>	Cex3	1	SEQ ID NO: 17	SEQ ID NO: 18	SEQ ID NO: 19	SEQ ID NO: 20
<i>C. exilicauda</i>	Cex4	1	SEQ ID NO: 21	SEQ ID NO: 22	SEQ ID NO: 23	SEQ ID NO: 24
<i>C. exilicauda</i>	Cex5	1	SEQ ID NO: 25	SEQ ID NO: 26	SEQ ID NO: 27	SEQ ID NO: 28
<i>C. exilicauda</i>	Cex6	1	SEQ ID NO: 29	SEQ ID NO: 30	SEQ ID NO: 31	SEQ ID NO: 32
<i>C. exilicauda</i>	Cex7	1	SEQ ID NO: 33	SEQ ID NO: 34	SEQ ID NO: 35	SEQ ID NO: 36
<i>C. exilicauda</i>	Cex8	1	SEQ ID NO: 37	SEQ ID NO: 38	SEQ ID NO: 39	SEQ ID NO: 40
<i>C. exilicauda</i>	Cex9	1	SEQ ID NO: 41	SEQ ID NO: 42	SEQ ID NO: 43	SEQ ID NO: 44
<i>C. exilicauda</i>	Cex10	1	SEQ ID NO: 45	SEQ ID NO: 46	SEQ ID NO: 47	SEQ ID NO: 48
<i>C. exilicauda</i>	Cex11	1	SEQ ID NO: 49	SEQ ID NO: 50	SEQ ID NO: 51	SEQ ID NO: 52
<i>C. limpidus limpidus</i>	ClI2b	1	SEQ ID NO: 53	SEQ ID NO: 54	SEQ ID NO: 55	SEQ ID NO: 56
<i>C. limpidus limpidus</i>	ClI3	1	SEQ ID NO: 57	SEQ ID NO: 58	SEQ ID NO: 59	SEQ ID NO: 60

Species	A	A1	B	C	D	E
C. limpidus limpidus	CII4	1	SEQ ID NO:61	SEQ ID NO:62	SEQ ID NO:63	SEQ ID NO:64
C. limpidus limpidus	CII5b	1	SEQ ID NO:65	SEQ ID NO:66	SEQ ID NO:67	SEQ ID NO:68
C. limpidus limpidus	CII5c	1	SEQ ID NO:69	SEQ ID NO:70	SEQ ID NO:71	SEQ ID NO:72
C. limpidus limpidus	CII6	1	SEQ ID NO:73	SEQ ID NO:74	SEQ ID NO:75	SEQ ID NO:76
C. limpidus limpidus	CII7	1	SEQ ID NO:77	SEQ ID NO:78	SEQ ID NO:79	SEQ ID NO:80
C. limpidus limpidus	CII8	1	SEQ ID NO:81	SEQ ID NO:82	SEQ ID NO:83	SEQ ID NO:84
C. noxius	Cn4b	1	SEQ ID NO:85	SEQ ID NO:86	SEQ ID NO:87	SEQ ID NO:88
C. noxius	Cn10b	1	SEQ ID NO:89	SEQ ID NO:90	SEQ ID NO:91	SEQ ID NO:92
C. elegans	Ce3	1	SEQ ID NO:93	SEQ ID NO:94	SEQ ID NO:95	SEQ ID NO:96
C. elegans	Ce5	1	SEQ ID NO:97	SEQ ID NO:98	SEQ ID NO:99	SEQ ID NO:100
C. elegans	Ce6	1	SEQ ID NO:101	SEQ ID NO:102	SEQ ID NO:103	SEQ ID NO:104
C. elegans	Ce6b	1	SEQ ID NO:105	SEQ ID NO:106	SEQ ID NO:107	SEQ ID NO:108
C. elegans	Ce7	1	SEQ ID NO:109	SEQ ID NO:110	SEQ ID NO:111	SEQ ID NO:112
C. elegans	Ce13	1	SEQ ID NO:113	SEQ ID NO:114	SEQ ID NO:115	SEQ ID NO:116
C. elegans	Ce13b	1	SEQ ID NO:117	SEQ ID NO:118	SEQ ID NO:119	SEQ ID NO:120
C. gracilis	Cg1	1	SEQ ID NO:121	SEQ ID NO:122	SEQ ID NO:123	SEQ ID NO:124
C. gracilis	Cg1b	1	SEQ ID NO:125	SEQ ID NO:126	SEQ ID NO:127	SEQ ID NO:128
C. gracilis	Cg2	1	SEQ ID NO:129	SEQ ID NO:130	SEQ ID NO:131	SEQ ID NO:132
C. gracilis	Cg3	1	SEQ ID NO:133	SEQ ID NO:134	SEQ ID NO:135	SEQ ID NO:136
C. sculpturatus	CsEv1d	1	SEQ ID NO:137	SEQ ID NO:138	SEQ ID NO:139	SEQ ID NO:140
C. sculpturatus	CsEv1c	1	SEQ ID NO:141	SEQ ID NO:142	SEQ ID NO:143	SEQ ID NO:144
C. sculpturatus	CsEv3b	1	SEQ ID NO:145	SEQ ID NO:146	SEQ ID NO:147	SEQ ID NO:148
C. sculpturatus	CsE1a	1	SEQ ID NO:149	SEQ ID NO:150	SEQ ID NO:151	SEQ ID NO:152
C. sculpturatus	CsEv2c	1	SEQ ID NO:153	SEQ ID NO:154	SEQ ID NO:155	SEQ ID NO:156
C. sculpturatus	CsEv2b	1	SEQ ID NO:157	SEQ ID NO:158	SEQ ID NO:159	SEQ ID NO:160
C. sculpturatus	CsEv2d	1	SEQ ID NO:161	SEQ ID NO:162	SEQ ID NO:163	SEQ ID NO:164
C. sculpturatus	CsEv1b	1	SEQ ID NO:165	SEQ ID NO:166	SEQ ID NO:167	SEQ ID NO:168
C. sculpturatus	CsEv1e	1	SEQ ID NO:169	SEQ ID NO:170	SEQ ID NO:171	SEQ ID NO:172
C. sculpturatus	CsEv2a	1	SEQ ID NO:173	SEQ ID NO:174	SEQ ID NO:175	SEQ ID NO:176
C. sculpturatus	CsE9b	1	SEQ ID NO:177	SEQ ID NO:178	SEQ ID NO:179	SEQ ID NO:180
C. sculpturatus	CsE9	1	SEQ ID NO:181	SEQ ID NO:182	SEQ ID NO:183	SEQ ID NO:184
C. sculpturatus	CsE8	1	SEQ ID NO:185	SEQ ID NO:186	SEQ ID NO:187	SEQ ID NO:188
C. sculpturatus	CsE3	1	SEQ ID NO:189	SEQ ID NO:190	SEQ ID NO:191	SEQ ID NO:192
C. sculpturatus	CsE1x	1	SEQ ID NO:193	SEQ ID NO:194	SEQ ID NO:195	SEQ ID NO:196
C. exilicauda	CexErg1	2	SEQ ID NO:197	SEQ ID NO:198	SEQ ID NO:199	SEQ ID NO:200
C. exilicauda	CexErg2	2	SEQ ID NO:201	SEQ ID NO:202	SEQ ID NO:203	SEQ ID NO:204
C. exilicauda	CexErg3	2	SEQ ID NO:205	SEQ ID NO:206	SEQ ID NO:207	SEQ ID NO:208
C. exilicauda	CexErg4	2	SEQ ID NO:209	SEQ ID NO:210	SEQ ID NO:211	SEQ ID NO:212

Species	A	A1	B	C	D	E
C. limpidus limpidus	Cll Erg1	2	SEQ ID NO:213	SEQ ID NO:214	SEQ ID NO:215	SEQ ID NO:216
C. limpidus limpidus	Cll Erg2	2	SEQ ID NO:217	SEQ ID NO:218	SEQ ID NO:219	SEQ ID NO:220
C. limpidus limpidus	Cll Erg3	2	SEQ ID NO:221	SEQ ID NO:222	SEQ ID NO:223	SEQ ID NO:224
C. limpidus limpidus	Cll Erg4	2	SEQ ID NO:225	SEQ ID NO:226	SEQ ID NO:227	SEQ ID NO:228
C. noxius	Cn Erg3	2	SEQ ID NO:229	SEQ ID NO:230	SEQ ID NO:231	SEQ ID NO:232
C. noxius	Cn Erg4	2	SEQ ID NO:233	SEQ ID NO:234	SEQ ID NO:235	SEQ ID NO:236
C. noxius	Cn Erg5	2	SEQ ID NO:237	SEQ ID NO:238	SEQ ID NO:239	SEQ ID NO:240
C. elegans	CeErg1	2	SEQ ID NO:241	SEQ ID NO:242	SEQ ID NO:243	SEQ ID NO:244
C. elegans	CeErg2	2	SEQ ID NO:245	SEQ ID NO:246	SEQ ID NO:247	SEQ ID NO:248
C. elegans	CeErg3	2	SEQ ID NO:249	SEQ ID NO:250	SEQ ID NO:251	SEQ ID NO:252
C. gracilis	CgErg1	2	SEQ ID NO:253	SEQ ID NO:254	SEQ ID NO:255	SEQ ID NO:256
C. gracilis	CgErg2	2	SEQ ID NO:257	SEQ ID NO:258	SEQ ID NO:259	SEQ ID NO:260
C. gracilis	CgErg3	2	SEQ ID NO:261	SEQ ID NO:262	SEQ ID NO:263	SEQ ID NO:264
C. sculpturatus	CsErg1	2	SEQ ID NO:265	SEQ ID NO:266	SEQ ID NO:267	SEQ ID NO:268
C. sculpturatus	CsErg2	2	SEQ ID NO:269	SEQ ID NO:270	SEQ ID NO:271	SEQ ID NO:272
C. sculpturatus	CsErg3	2	SEQ ID NO:273	SEQ ID NO:274	SEQ ID NO:275	SEQ ID NO:276
C. sculpturatus	CsErg4	2	SEQ ID NO:277	SEQ ID NO:278	SEQ ID NO:279	SEQ ID NO:280
C. sculpturatus	CsErg5	2	SEQ ID NO:281	SEQ ID NO:282	SEQ ID NO:283	SEQ ID NO:284

A. Name given to the clone by the inventors

A1. Type of channel that modifies the native toxin, according to information on homologous toxins:

1. Sodium channels
2. Erg type potassium channels (ether a go go)

B. Sequence number of the complete clone

C. Number of the amino acid sequence encoded by the complete clone B

D. Number of the nucleotide sequence of the coding fragment of the mature peptide (same primary sequence as the native toxin)

E. Number of the amino acid sequence encoded by fragment D

[0025] Naturally occurring toxins in the scorpion venom will raise specific antibodies in a stung individual. In certain embodiments of the invention, the antibodies are specific against only a particular polypeptide and/or the toxin from a particular *Centruroides* species. Such polypeptides may be used as

part of a composition, where the polypeptide is bound covalently or through hydrophobic or hydrophilic interaction to a substrate. The substrate may then be used as part of a diagnostic device.

[0026] Another embodiment of the invention comprises a device using the substrate described in the previous paragraph. Such a device may be used to detect the presence of species-specific antibodies in an individual stung by a species of *Centruroides* scorpion. A method to diagnose whether the scorpion that stung an individual belongs to a particular species of *Centruroides* scorpion comprises contacting such a diagnostic device with a sample from a stung individual, and detecting the presence of antibodies from the individual that had been stung by the scorpion. If present, the antibodies raised against a particular naturally occurring toxin will bind to the polypeptides of the device. The bound antibodies can then be detected by methods and optical detecting systems well-known to those of ordinary skill in the art. Such methods and devices may for example, be based on immuno-enzymatic, immuno-fluorescent or immuno-chromatographic techniques.

[0027] A significant amount of toxins and their genes including the respective signal peptide are now known. From comparative analyses of the nucleotide and/or amino acid sequences, it can be seen that there are some highly conserved regions, like those equivalent to the first 6 amino acids of the signal peptide and part of the 3' uncoding region (UTR). Based on the foregoing, it is possible to design degenerate oligonucleotides corresponding to the amino ends of the signal peptide and the carboxyl end of the toxin, which can be amplified by PCR using the messenger RNA present in the telson of the scorpion in question. The sequences that hybridize with said oligos generate clones that comprise the coding sequence of the toxin and its peptide signal. These are cloned in a useful vector for rapid identification, as is the case of the vector PKS- (Stratagene, La Jolla, CA, USA) that has Beta galactosidase as marker in such a way that when X-gal is present in the solid culture medium the colonies that received inserts (clones) lose Beta galactosidase activity and grow with a white color, while this enzyme remains intact in the colonies that

received no insert and generates blue colonies. The white colonies are cultivated in order to amplify their plasmid DNA (that presumably comprises some of the clones of interest) which is subsequently sequenced to determine the nucleotide sequence and the deduced amino acid sequence.

[0028] Another alternative for oligonucleotide design is to purify some of the toxins present in the scorpion venom in question and obtain the amino acid sequence of the amino region (at least the first 8 amino acids) and use them to design specific oligonucleotides with which to try to obtain, in particular, the clones of these toxins, or rather, based on the homology between the amino acid sequences obtained, design a degenerate oligonucleotide to try to obtain the clones present in the telson that are homologous to the oligonucleotide that has been designed. In both cases, the clones obtained will comprise the coding sequence of the just the toxin without the signal peptide.

[0029] Another strategy to be used should there be no further information on the possible expected clones consists of using, instead of an oligonucleotide corresponding to the 3' region of the clone, a poly T oligonucleotide that should hybridize with the PolyA site present in all cDNAs independently of the direct oligo used, the one from the amino region of the signal peptide or of the mature peptide.

[0030] The choice of the strategy to be followed in each case will depend on the elements available such as the knowledge of the total or partial amino acid sequence of the toxins of interest or an analysis by mass spectrometry of one or more toxins of the venom of the scorpion in question, the sequence of other toxins from the same scorpion or from other related scorpions, the information on the signal peptide toxin sequence of the same scorpion or other related scorpions, to name but a few.

[0031] The peptide or toxin purification procedures using raw venoms from the different scorpions can begin with chromatographic columns that separate fractions based on screening of molecular mass, followed by ion exchange resins and HPLC using reverse phase columns. At present, most laboratories use direct separations of peptides by HPLC or use it directly after a simple

separation by molecular mass of the soluble venoms (Possani, L.D., *et al.*, *Eur. J. Biochem.* 264:287-300 (1999)). To this end, the whole venom of the scorpion in question is obtained by electrostimulation of the telson and is subsequently centrifuged before beginning the purification processes.

[0032] It is known that some of the polypeptide precursors of the toxins suffer posttranslational modifications in both the amino and carboxyl ends: in the amino end a signal peptide of 18 to 21 amino acids is normally eliminated by means of a signal peptidase. In the carboxyl end, the extra basic amino acids (Arg and/or Lys) are processed by a carboxypeptidase. Several mechanisms have been discovered for the posttranslational processing of the carboxyl end, typically the basic residue(s) of the end are removed. When a glycine residue precedes one or two basic residues of the amino group of the glycine residue, it is used to amidate to the amino acid residue that precedes it. If a glycine precedes a group of three basic residues, the basic triad is removed without there being any amidation (Possani, L.D., *et al.*, *Eur. J. Biochem.* 264:287-300 (1999)). It will therefore be appropriate if the recombinant peptides being produced have a primary sequence the most similar possible to the mature toxin, that is, without the signal peptide and without the amino acids of the carboxyl end that are eliminated when the toxin is processed, when this is the case.

[0033] In order for the genes or clones of the present invention to be used in the generation of better antivenoms, they should first be expressed in a heterologue system, as for example *Escherichia coli*, *Pichia pastoris*, *Baculovirus* or others, for either the corresponding recombinant peptide or a fusion protein comprising said peptide to then be used as immunogen (antigen) or component of an immunogen for the production of antibodies in mammals.

[0034] It is clear that in order to be expressed in a heterologue system, the genes or clones of the present invention should be introduced in genetic constructions that are compatible with the expression in said systems. Some examples of these could be the Protein Expression and Purification System of

New England Biolabs where plasmid pMal-C is used in *E. coli* to express the proteins of interest as fusion proteins bound to the maltose binding protein. Another system could be the one used by Legros' group (Legros, C., *et al.*, *Vaccine* 20:934-942 (2002)), pMal-p from the same supplier.

[0035] Before inserting the genes or clones of the present invention in said genetic constructions, it is necessary to edit them to eliminate any fragment of 3' and 5' uncoding regions (UTR). In order to edit the genes or clones of the present invention, it is necessary to synthesize oligonucleotides that, for the direct oligonucleotide, comprise the first amino acids (from 6 to 9) of the amino terminal region to be expressed, either the signal peptide, if it is to be included in the expressed protein, or the mature peptide if the signal peptide is to be excluded and, for the reverse oligonucleotide, the last 6-9 amino acids of the carboxyl terminal region of the mature peptide. In the cases of those toxins that are known to be postrtranslationally processed, it will be convenient for the reverse oligo to be designed leaving out the amino acid residue(s) that are known to be eliminated with the processing. Subsequently, using both oligonucleotides as primers and the clone or gene of interest as template, a PCR amplification reaction is performed to obtain the DNA that codes only for the mature peptide or for the mature peptide plus the signal peptide. It can also be recommendable to take advantage of the direct oligo to include a methionine residue just before the first amino acid. This, at some moment, will permit cleavage of the recombinant peptide once the fusion protein has been expressed through the application of cyanogen bromide (Possani, L.D., *et al.*, *Biochem. J.* 229:739-750 (1985))

[0036] In order to demonstrate the feasibility of using any of the genes or clones isolated from scorpions of the genus *Centruroides*, including those of the present invention, for industrial production of the recombinant peptide whose primary sequence is identical to that of the encoded toxin, that is, the native toxin, either in free form or fused with part of other proteins producing a larger polypeptide, the inventors of the present invention carried out a construction (shown in figure 1) using clone CngtII (Becerril, B., *et al.*, *Gene*

128:165-171 (1993)), that codes for a well-known, characterized toxin of *Centruroides noxius* Hoffmann, Cn5 (García, C., *et al.*, *Com. Biochem. Physiol. 116B (3)*:315-322 (1997)), in which this clone was fused to the maltose binding protein. This is illustrated in detail in examples 8 and 9. The fusion protein expressed was used to generate antibodies in mammals as can be seen in example 10, while in example 11 the use is illustrated of said antibodies in the neutralization in vivo of a known toxin specific for mammals whose amino acid sequence is similar to that of Cn5 and which has proved to be one of the most important in its toxic effect, Cn2 (Zamudio, F., *et al.*, *Eur. J. Biochem. 204*:281-292 (1992); García, C., *et al.*, *Com. Biochem. Physiol. 116B (3)*:315-322 (1997)).

[0037] Any of the clones of the present invention can, like CngtII, be edited by designing specific oligos which, as mentioned earlier, can be used for the insertion of some methionine or some other sequence that permits its purification, for example, by amplifying them by PCR using the clone of choice as template, obtaining DNA fragments that comprise the corresponding sequence reported in column D, Table 1. Constructions can be made with said fragments in commercial systems, such as plasmids pMalC and pMal-p of the Protein Expression and Purification System (New England Biolabs), or in manufactured expression systems that comprise said DNA fragments fused to heterologue protein coding fragments or fragments of the same, transforming hosts into bacteria such as the CMK strain of *Escherichia coli* or any other expression host for which the selected expression system is appropriate.

[0038] On cultivating said cells of the recombinant host, these cells will express (after induction) the fusion protein that will comprise the corresponding sequence reported in column E, Table 1.

[0039] It is known that the genetic code is degenerate, that is, that for one same amino acid there is generally more than one encoding codon. The difference between these codons is the third of the bases. It is obvious to an expert in the state of the technique that it is possible to substitute some bases in the encoding nucleotide sequence of the clones of the present invention

referred to in column B or in the edited sequences of column D, Table 1, that encode exactly the same amino acid sequences as those referred to in column E, Table 1. This may be particularly useful when it is wished to express said peptides of the present invention in different recombinant hosts, for it is known that different types of hosts have a "preference" of use towards certain codons for determined amino acids. Such "silent mutations" fall within the scope of the present invention, since the products of their expression are again the peptides referred to in column E, Table 1, of the present invention.

[0040] Recombinant peptides for the present invention shall be understood to be those peptides obtained by recombinant methods that comprise the primary sequence reported in column E, Table 1.

[0041] Thus, the present invention also refers to the use of the recombinant peptides of the present invention, either free or as part of fusion proteins, as vaccines to prevent envenomation from the venom of scorpions of the genus *Centruroides* and the pharmaceutical preparations of said vaccine. Administration of the peptides may be by intravenous, subcutaneous, intramuscular, intravaginal, intraperitoneal, intranasal, oral or other mucous routes. Additionally, the hyperimmune sera or antibodies (obtained following injection of the polypeptides of the invention) that can neutralize or delay the toxic effect of the scorpion toxins can be used to treat envenomation (serotherapy).

[0042] The vaccines of the present invention comprise one or more of the recombinant peptides of the present invention, either free or as fusion proteins that, in turn, comprise the primary sequence of the peptides of the present invention. Since the folding of the recombinant peptides expressed in heterologue hosts is not the same as that of the native toxin, said vaccine is also sufficiently innocuous to be administered without danger of intoxication, it is stable and compatible with vaccine carriers.

[0043] An effective amount of the vaccine should be administered that is capable of producing an immune response in a mammal, where "effective amount" is defined as an amount of recombinant peptides from the present

invention or any fusion protein comprising the same. The necessary amount will vary depending on whether the peptides of the present invention are used or fusion proteins comprising these peptides and on the antigenicity of said fusion protein and on the species and weight of the subject to be vaccinated, but it can be estimated by standard techniques.

[0044] Pharmaceutically useful compositions can be formulated as vaccines that comprise one or more of the recombinant peptides of the present invention or any fusion protein including said peptides, according to known methods such as the addition of a pharmaceutically acceptable carrier. In order to form a pharmaceutically acceptable composition suitable for effective administration, said composition shall contain an effective amount of one or more of the recombinant peptides of the present invention or any fusion protein including said recombinant peptides.

[0045] The pharmaceutical compositions of the vaccines of the present invention can include a pharmaceutically acceptable adjuvant such as aluminum or calcium gels, modified muramyl dipeptides, monophosphorylated lipids, liposomes, delayed release capsules, polyglycolic acids and polyamino acids. Polyglycolic and polyamino acids are also useful for the oral administration of vaccines. Some examples of aluminum gels useful as adjuvants include precipitated aluminum salts such as aluminum phosphate and hydroxide. Some preservatives such as thimerosal, dextrane and glycerine can be added to stabilize the final vaccine. If it is wished to have the vaccines in injectable form, immunologically acceptable diluents or carriers can be included.

[0046] The vaccine of the present invention or the pharmaceutical compositions of the same can be administered to mammals locally and/or systemically through the conventional routes such as the intravenous, subcutaneous, intramuscular, intravaginal, intraperitoneal, intranasal, oral or other mucous routes to arouse an efficacious immune response to protect against the venom of scorpions of the genus *Centruroides*. The vaccine can be

optionally administered in sole or multiple doses with the object of sustaining antibody levels.

[0047] The pharmaceutic compositions of the vaccines of the present invention should be administered to an individual in such amounts that they contain effective amounts of the vaccine of the present invention. The effective amount will vary according to a variety of factors such as species, condition, weight, sex and age of the individual to be treated. Another factor includes the administration route used.

[0048] Another scope of the present invention is based on the fact that the recombinant peptides of the present invention, either free or as fusion proteins, can also be used to generate an immunogenic matrix when bound either covalently or through hydrophobic or hydrophilic interactions to some substrate like polyacrylamide, polyvinyl, activated aldehyde agarose (US patents Nos. 5,904,922 and 5,443,976), sepharose, carboxymethyl cellulose or some other, in such a way that the matrix is capable of specifically binding either antibodies (raised against the whole venom of scorpions of the genus *Centruroides* or against the same venoms enriched with some of the recombinant peptides of the present invention, or against mixtures of recombinant peptides of the present invention) or the F(ab) or F(ab)₂ fragments obtained from hydrolysis of said antibodies, and is useful in the purification by immunoaffinity of said antibodies or F(ab) or F(ab)₂ fragments, which is why said use in the antigenic matrix and said antigenic matrix are included in the scope of the present invention.

Materials and Methods

[0049] Scorpions of the species *Centruroides exilicauda* were collected in Baja California, Mexico. Only 2 animals were used for clone isolation.

[0050] Scorpions of the species *C. limpidus limpidus* Karsh were collected in Guerrero, Mexico. Only 5 animals were used for clone isolation.

[0051] Scorpions of the species *C. noxius* Hoffmann were collected in Nayarit, Mexico. Only 1 animal was used for clone isolation.

- [0052] Scorpions of the species *C. elegans* were collected in Jalisco, Mexico. Only 5 animals were used for clone isolation.
- [0053] Scorpions of the species *C. gracilis* were collected in Veracruz, Mexico. Only 1 animal was used for clone isolation.
- [0054] Scorpions of the species *C. sculpturatus* Ewing were collected in Tucson Arizona. Only 5 animals were used for clone isolation.
- [0055] All the reagents used are of an analytical grade.

Obtaining the scorpion venom

- [0056] The venom of each scorpion was obtained by electrostimulating the telson. The mixture of the venom from all the scorpions of the same species was centrifuged at 10,000g for 15 min. The supernatant was quantified by absorbance at 280 nm, lyophilized and stored at -40°C until it was used.

Purification of toxins from the venoms

- [0057] The toxins were purified in three sequential chromatographic steps:
- i) In the first place, using a molecular filter in a medium Sephadex G-50 column (Amersham Pharmacia Biotech AB, Uppsala Sweden). Columns of 200 X 0.9 cm were used with a 30ml/hr flow of 20 mM ammonium acetate Buffer pH 4.7 for approximately 10 hr.
 - ii) The different fractions of interest obtained in the preceding step were separated by ion exchange in 30X0.9 cm columns packed with CMC-32 (Whatman, England). They were run for approximately 15 hr with a 30ml/hr flow of ammonium acetate buffer pH 4.7 in a gradient of 0.05 M NaCl , applying 250 ml on each side.
 - iii) The fractions of interest obtained from the preceding step were subsequently separated by HPLC in a C-18 reverse phase analytic column (Vidac, Hesperia, CA, USA), with 0-60% solution B gradient for 60 min with a flow of 1ml/min. Solution A is water with 0.12% trifluoroacetic acid (TFA) (TFA) (Pierce, Rockford, IL, USA) and solution B is acetonitrile (Pierce,

Rockford, IL, USA) with 0.10% TFA. The apparatus used was a Waters (Millipore Co., Milford, MA, USA) model 625 LC System with a Waters 996 Diode Photoarray Detector.

The readings of the different eluted fractions from Sephadex G-50 and CMC-32 were read in a Beckman DU-50 spectrophotometer at 280 nm.

Partial or total sequencing of peptides

- [0058] Peptide sequentiation was performed following the automatic Edman degradation method (Edman, P. and Begg, G., *Eur. J. Biochem.* 1:80-91 (1967)) with a Beckman LF300 sequencer.

Isolation of clones from scorpion toxins

- [0059] The RNAm from the poisonous glands (telsons) of the scorpions of each species was isolated using the method of Chirgwin et al. (1979). Total RNA (approximately 500 ng) was used to synthesis cDNA through the use of an oligonucleotide which is a 22mer polyT22NN, followed by two degenerate nucleotides (N). Synthesis of the first chain was performed in a first chain 1X buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM MgCl₂), 10mM dithiothreitol (DTT), 0.5mM dNTPs, 200 units of reverse transcriptase M-MLV (Gibco-BRL, Gran Islands, New York, USA), 0.5mM oligo (T)22NN, RNase inhibitor units (Boehringer Mannheim, Frankfurt, Germany), in a final volume of 20 ml. The mixture was pre-incubated for 5 min at 65°C, and then for 5 min. at 50°C and 30 min. at 42°C. The DTT and enzyme were added to the reaction when the mixture reached 42°C, just before the final 30 min.

- [0060] For the polymerase chain reaction (PCR), a sample was taken of the first chain reaction (2ml) to which was added a Vent DNA polymerase 1X buffer (10mM KCl, 10 mM (NH₄)₂ SO₄, 20 mM Tris-HCl, pH 8.8, 2mM MgSO₄, 0.1% Triton X-100, at 25°C), 200mM dNTPs, 0.25 mM of the direct oligonucleotide (in the 5'-3' sense), 0.25 mM of the reverse oligonucleotide (in the 3'-5' sense) and two units of Vent DNA Polymerase (New England

Biolabs, Beverly MA, USA) in a final volume of 50 ml. The reaction was carried out using a Perking Elmer 9600 thermocycler with the following protocol: Incubation of the mixture for 3 min at 94°C, 5 min a 55°C before adding the enzyme, followed by 30 s at 72° for the first cycle. The mixture was then incubated at 94°C for 30 s followed by 30 s at 52°C per cycle and 30 s at 72°C per cycle, and repeated 32 times before a final step of 10 min at 72°C.

[0061] The PCR products were purified in a Centricon 100 column (Amicon, Beverly, MA, USA) following the manufacture's instructions. They were subsequently bound to the EcoRV site of plasmid PKS-. These constructions were used to transform E. coli DH5-alpha cells. The selection of clones that comprised some insert was done by plating the transforming cells in Petri dishes with LB/agar in the presence of X.Gal, choosing the white colonies for plasmid amplification. The plasmid DNA were sequenced in both chains using fluorescent nucleotides in a Perkin Elmer Applied Biosystems apparatus (Foster City, CA, USA) as described by the manufacturer.

[0062] In order to better illustrate how the clones or genes and recombinant peptides of the present invention were obtained and their modes of use, the following specific examples are provided to better help the reader in the different aspects of the practice of the present invention. Given that these specific examples are simply illustrative, in no case should the following descriptions be considered as limiting the scope of the following invention:

EXAMPLES

EXAMPLE 1

Isolation and cloning of the genes of *C. exilicauda* toxins

[0063] The venom was processed as indicated in the materials and methods section in order to purify toxins and the amino region of the isolated toxins was sequenced as described in the materials and methods section.

[0064] The first chain was then obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was initially oligonucleotide D1 which is a 25mer with the sequence 5'-gagatgaattcgttggtgatgata-3' (SEQ ID NO: 286) and R1 as reverse oligonucleotide with sequence 5'-gcaattaagaagcgttacaata-3' (SEQ ID NO: 287).

[0065] Five clones were obtained with this first strategy (first amplification reaction) (SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 13 and SEQ ID NO: 17), four of which, as well as the coding sequence of the mature peptide, also presented the coding sequence of the complete sequence of the signal peptide and one of them only included the sequence corresponding to the carboxyl region of the signal peptide. The number of clones isolated with this first amplification reaction proved to be less than the number of toxins directly isolated from the venom, and it was thus decided to design another direct oligonucleotide whose sequence is 5'-gmaarggarggtatc-3' (SEQ ID NO: 288), benefiting from the fact that all the signal peptides of known toxins finish with an Ala in the carboxyl end and from the homologies between the amino regions of the toxins that had already been partially sequenced.

[0066] A further amplification reaction was then performed using this new oligonucleotide and 5 additional clones were found (SEQ ID NO: 21, SEQ ID NO: 25, SEQ ID NO: 29, SEQ ID NO: 33 and SEQ ID NO: 37), all of which showed sequences corresponding to the mature peptide and only the last amino acid of the signal peptide.

[0067] A new amplification reaction was performed with a third oligo 5'-raaggasggt tatccb -3' (SEQ ID NO: 289) and three additional clones were obtained (SEQ. ID. No: 41, SEQ ID NO: 45 and SEQ ID NO: 49).

EXAMPLE 2

Isolation and cloning of the genes of the sodium toxins from *C. limpidus* *limpidus*.

[0068] The first chain was obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was initially the same D1 oligonucleotide (SEQ ID NO: 286) and R1 as reverse oligonucleotide (SEQ ID NO: 287).

[0069] With this first strategy (first PCR), 7 clones were obtained (SEQ ID NO: 53, SEQ ID NO: 57, SEQ ID NO: 61, SEQ ID NO: 65, SEQ ID NO: 69, SEQ ID NO: 73 and SEQ ID NO: 77) with the complete coding sequence of the signal peptide and the mature peptide. In this case, a genomic clone was also obtained (SEQ ID NO: 81) whose complete sequence could not be sequenced clearly, and hence only the coding sequence of the last 4 amino acids of the carboxyl region of the signal peptide plus the complete mature peptide are presented. The presence of genomic clones in whole RNA preparations is not common. It can only be explained if the genomic DNA was not completely removed from the preparation, as could be the case here.

EXAMPLE 3

Isolation and cloning of the genes of the sodium toxins of *C. noxius* Hoffmann.

[0070] The first chain was obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was initially the same oligonucleotide D1 (SEQ ID NO: 286) and R1 as reverse oligonucleotide (SEQ ID NO: 287).

[0071] Two clones were obtained with this strategy (SEQ ID NO: 85 y SEQ ID NO: 89,), with the complete coding sequence of the signal peptide and mature peptide.

EXAMPLE 4

Isolation and cloning of the genes of the sodium toxins of *C. elegans*

[0072] The first chain was obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was the same oligonucleotide D1 (SEQ ID NO: 286) and R1 as reverse oligonucleotide (SEQ ID NO: 287).

[0073] Seven clones were obtained with this strategy (SEQ ID NO: 93, SEQ ID NO: 97, SEQ ID NO: 101, SEQ ID NO: 105, SEQ ID NO: 109, SEQ ID NO: 113 and SEQ ID NO: 117), all of which had the complete coding sequence of the signal peptide and the mature peptide.

EXAMPLE 5

Isolation and cloning of the genes of the sodium toxins of *C. gracilis*.

[0074] The first chain was obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was the same oligonucleotide D1 (SEQ ID NO: 286) and R1 as reverse oligonucleotide (SEQ ID NO: 287).

[0075] Four clones were obtained with this strategy (SEQ ID NO: 121, SEQ ID NO: 125, SEQ ID NO: 129 and SEQ ID NO: 133), all of which had the complete coding sequence of the signal peptide and the mature peptide.

EXAMPLE 6

Isolation and cloning of the genes of the sodium toxins of *C. sculpturatus*.

[0076] The first chain was obtained and amplified by PCR as mentioned in the materials and methods section. The direct oligonucleotide used in this case was the same oligonucleotide D1 (SEQ ID NO: 286) and R1 as reverse oligonucleotide (SEQ ID NO: 287).

[0077] Fifteen clones were obtained with this strategy (SEQ ID NO: 137, SEQ ID NO: 141, SEQ ID NO: 145, SEQ ID NO: 149, SEQ ID NO: 153, SEQ ID NO: 157, SEQ ID NO: 161, SEQ ID NO: 165, SEQ ID NO: 169, SEQ ID NO: 173, SEQ ID NO: 177, SEQ ID NO: 181, SEQ ID NO: 185, SEQ ID NO: 189 and SEQ ID NO: 193), all of which had the complete coding sequence of the signal peptide and mature peptide.

EXAMPLE 7

Isolation and cloning of the genes of the ERG type potassium toxins of 6 species of scorpions of the genus *Centruroides*.

[0078] The first chain was obtained and amplified in two PCR reactions for each one of the species of scorpions separately, as mentioned in the materials and methods section. The direct oligonucleotide used in this case was 5'-gtagagatagctgtgttgataaatca-3' (SEQ ID NO: 292) and as reverse oligonucleotide 5'-mmtaatctttattttc-3' (SEQ ID NO:290) for one of the reactions and ErgR2 5'-aattgcggaaattmm-3' (SEQ ID NO:291) for the other.

[0079] Four clones of *C. exilicauda* were obtained from both reactions (SEQ. ID. NO: 197, SEQ ID NO: 201, SEQ ID NO: 205 and SEQ ID NO: 209); 4 clones of *C. limpidus limpidus* (SEQ. ID. NO: 231, SEQ ID NO: 217, SEQ ID NO: 221 and SEQ ID NO: 225); 3 clones of *C. noxius* (SEQ. ID. NO: 229, SEQ ID NO: 233 and SEQ ID NO: 237); 3 clones of *C. elegans* (SEQ. ID. NO: 241, SEQ ID NO: 245 and SEQ ID NO: 249); 3 of *C. gracilis* (SEQ. ID. NO: 253, SEQ ID NO: 257 and SEQ ID NO: 261); and 5 clones of *C. sculpturatus* (SEQ. ID. NO: 265, SEQ ID NO: 269, SEQ ID NO: 273, SEQ ID NO: 277 and SEQ ID NO: 281).

EXAMPLE 8

Genetic construction for the heterologue expression of a fusion protein of a toxin of a scorpion of the genus *Centruroides*.

[0080] Clone CngtII that codes for the toxin Cn5 was obtained from a cDNA library as described in Becerril et al., 1993. In order to edit it, two specific oligonucleotides were designed and synthesized, direct oligo 5'-atgaaagaaggttatctggtaaac-3' (SEQ. ID. NO:293), that comprises amino acids 1 to 7 of the Cn5 toxin, permitted removal of the signal peptide and the inclusion of a methionine codon just before amino acid 1 in order to permit cleavage of peptide Cn5 with cyanogen bromide after its expression, permitting release of the recombinant peptide using the expressed fusion protein. The reverse oligo 5'-ttagctgcaagattattaggaag-3' (SEQ. ID. NO:294) was designed to eliminate the last two amino acids that are not present in the mature Cn5 toxin (Lys 67 and Lys 68). The DNA coding for peptide Cn5 was amplified by PCR using clone CngtII as template and the designed oligos as primers. This DNA was bound to a plasmid pMalC XmaI site of the Protein Expression and Purification System (New England Biolabs), which has a specific factor Xa site that permits enzyme cleavage of the fusion protein. Figure 1 shows the generated construction in which the peptide Cn5 sequence was confirmed by sequencing both chains.

EXAMPLE 9

Heterologue expression of the fusion protein comprising recombinant peptide Cn5

[0081] Once the inserts (DNA coding for peptide Cn5) had been bound to plasmid pMalC as in the above example, they were transformed into *Escherichia coli* strain CMK (Sambrook, J., et al., "*Molecular cloning a laboratory manual*." Second Edition, Cold Spring Harbor Laboratory Press, New York (1989)). Five ml of cultures were grown overnight in 500 ml of Luria Broth supplemented with 2.5 g glucose and 200 mg/ml ampicillin. The expression of the recombinant protein was induced when the culture reached an absorbance value of 0.5 to 600 nm, and it was harvested 3.5 hr. later. The

cells were processed and the fusion protein was purified using affinity chromatography, following the supplier's protocol.

[0082] The fusion protein (FP) was expressed in the cytoplasm of the *E. coli* cells, comprising protein sequence Mal E (maltose binding protein) plus peptide Cn5 sequence. After extraction of cytoplasm from the culture cell pack, the expression yield of the FP was in the order of 50mg/L. The presence of hybrid FP was shown using SDS PAGE where the presence of the product with the expected molecular mass was observed (lane 4, figure 2, where the product can be seen after being purified by affinity). After digestion with factor Xa, proteins can be observed corresponding to the size expected of the maltose binding protein and the size of the Cn5 (see lane 5, figure 2).

[0083] Specific recognition assays were conducted by immunoblot using antibodies generated against native toxin Cn5 as in example 10. This is shown in figure 3. Lanes 3 and 4 present a positive recognition of the FP comprising peptide Met-Cn5, while in lane 5, where the FP was applied after being digested with factor Xa, only peptide Met-Cn5 is recognized, contrary to Mal E. Lane 6 shows native toxin Cn5 as control, which is clearly recognized by the antibodies.

[0084] Peptide Met-Cn5 was purified by HPLC (data not shown) and the peptide was sequenced by automatic Edman degradation unequivocally confirming the first 10 amino acids, including the extra methionine. The final yield of expression of clone CngtII (peptide Met-Cn5) was calculated at 5mg/L.

[0085] In this way the feasibility was proved of using any clone or isolated gene from the telson of scorpions of the genus *Centruroides*, including those of the present invention, for their heterologue expression, thus obtaining either a fusion protein comprising the primary sequence of the toxin encoded by the gene or clone used or a recombinant peptide comprising said sequence, and that said product is able to specifically bind antibodies generated against the native toxin (encoded by the gene in question), which can be used to purify specific antibodies to that toxin using a mixture of antibodies generated

against a mixture of several toxins, among which the native toxin (encoded by the gene in question) can be found, as could be the case of the whole venom of a scorpion of the genus *Centruroides* or mixtures of venoms of more than one scorpion.

EXAMPLE 10

Use of a fusion protein comprising a primary sequence identical to that of a native toxin of a scorpion of the Genus *Centruroides*, as immunogen in rabbits for the development of polyclonal antibodies.

[0086] The fusion protein (FP) obtained in the above example was used as immunogen for the development of polyclonal antibodies in rabbits. To this end, female new Zealand rabbits were used (2kg starting weight). 100mg of FP or the native toxin Cn5 were applied in Freund's complete adjuvant (1 ml) for the first dose. The 3 subsequent doses were applied in Freund's incomplete adjuvant (1 ml) every 15 days. All the applications were subcutaneous. The rabbits were bled 9 days after the fourth immunization to obtain the sera.

[0087] The sera were titered by ELISA, to which end Costar plates with 96 wells were covered with 150 ng per well of fusion peptide (FP) dissolved in a 120 mM sodium bicarbonate buffer pH 9.5 overnight at 4°C. Subsequently, the plates were blocked with 3% bovine serum albumin (BSA) in a PBS buffer (150mM sodium chloride and 15 mM sodium phosphate, pH7.4) for 1 hr at 37°C. Two-fold serial dilutions were prepared for the serum beginning with a 1:50 dilution with a PBS buffer containing Tween-20 0.1% and BSA 1%.

[0088] After incubating for 2 hr. at 37°C, the plates were washed in a washing solution (0.1%Tween-20 in PBS). Anti-rabbit goat antibodies were used coupled to horse-radish peroxidase (Biorad, Hercules, CA) as second antibodies. After 1 hour at 37°C, the plates were washed and ortho-phenylenediamine plus hydrogen peroxide were added as substrates. The enzyme reaction was stopped by the addition of sulfuric acid 4N. Absorbance

was monitored at 492 nm in a microplate reader (EIA model 1550, Biorad, Hercules, CA). Preimmune rabbit serum was used as control.

[0089] As can be seen in figure 4, the fusion protein produced antibodies that recognized the same FP in an excellent way in comparison to the antibodies generated against native toxin Cn5.

EXAMPLE 11

Use of the antibodies generated against the fusion protein that comprises the amino acid sequence of peptide Cn5 to neutralize a specific toxin for mammals in vivo.

[0090] As mentioned earlier, toxin Cn2 is one of the principal toxins of the venom of *C. noxius* Hoffmann responsible for the intoxication of mammals and has similarities at primary sequence level with Cn5. The antibodies generated against native Cn5 and against the fusion protein, both generated in rabbits as shown in example 10, to neutralize toxin Cn2 were therefore tested.

[0091] To this end, several values of the LD50 of toxin Cn2 were mixed with 250 ml of both immune (anti-Cn5 and anti-FP) and preimmune (taken before beginning the immunization scheme) (control) rabbit serum and were incubated at room temperature for 1hr and gently stirred. After this period, non-immune CD1 mice, 8 weeks old, were injected subcutaneously with the mixtures and their rates of survival 24 hr after the challenge were recorded (See Table 2).

TABLE 2. Survival rates in mice challenged with toxin Cn2 incubated with the sera

LD50 of Cn2	Serum	Survival Rate (live/total)	Percentage
1.0	Pre-immune	0/6	0
1.0	Anti-Cn5	7/7	100
1.5	Anti-Cn5	7/7	100
2.0	Anti-Cn5	6/7	86
1.0	Anti-FP	5/6	83

1 LD50 of Cn2= 0.6 µg/20 g (Licea, A.F., *et al.*, *Centruroides noxius Hoffmann. Toxicon* 34:843-847 (1996)).

[0092] The anti-Cn5 antibodies proved to have total protection capacity in the challenged mice with up to 2.5 LD50 of Cn2, while the anti-FP antibodies proved to have a reasonable protective capacity with 1 DL50. These results are especially significant considering that the mice challenged with a DL50 preincubated with pre-immune serum should have had a survival rate of 50%, however for this experiment in particular it worked with DL 100 (all the control mice died). This effect has been observed in the laboratory when a recently prepared toxin is used. Meanwhile, observe the challenge experiment (table 2) on the same day under the same conditions with mice from sibling litters.

SUMMARY

[0093] The present invention refers to 71 clones of scorpions of the genus *Centruroides* that code for toxins affecting sodium and Erg type potassium channels; to genetic constructions that comprise the coding fragment of said toxins; the recombinant proteins that include in their sequence the sequence of the mature peptides encoded by the clones of the present invention; and their uses as immunogens or antigens for the generation of specific antibodies in mammals; as part of an immunogenic matrix for fractionating by immunoaffinity the antivenoms currently produced; and as a vaccine.

[0094] Similarly, the present invention also refers to expression vectors that comprise the coding sequence of the toxins of the present invention, to the recombinant hosts comprising said vectors, and to the methods used for their expression.

[0095] All publications, patents and patent publications cited herein are incorporated by reference in their entirety into the disclosure. The foregoing specification, including the specific embodiments and examples, are intended

to be illustrative and not limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.